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Karolinska Institutet, Stockholm, Sweden

# **HOST SUSCEPTIBILITY TO ROTAVIRUS INFECTION AND DEVELOPMENT OF ANTIBODY- BASED IMMUNOTHERAPY**

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# HOST SUSCEPTIBILITY TO ROTAVIRUS INFECTION AND DEVELOPMENT OF ANTIBODY-BASED IMMUNOTHERAPY

THESIS FOR DOCTORAL DEGREE (Ph.D.)

by

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“Somewhere, something incredible is waiting to be known.”

Carl Edward Sagan

*Hayranlık duyduğum, tanıdığım en güçlü ve en sevgi dolu kadına,*

*Anneme...*

To my mother, the strongest woman I have ever known.

I admire and love you.



# ABSTRACT

Rotavirus infects mature enterocytes of the small intestine of young children and cause gastroenteritis, leading to approximately 500 000 deaths annually worldwide, 85 % of which occur in the developing world. The main objectives of the thesis were to investigate host genetic factors leading to differential susceptibility to rotavirus infections and to develop an antibody-based oral therapy against the infections.

Reduced *TLR3* expression was previously suggested to be associated with susceptibility to rotavirus infections. In **Paper I**, we thus investigated rotavirus-specific IgG antibody responses from individuals (IgA competent or deficient) using two *TLR3* SNPs (rs3775291 and rs5743305). We concluded that these two polymorphisms were associated with elevated IgG titers in IgA deficient, but not in IgA competent individuals. In addition, recent *in vitro* studies have suggested that HBGAs (H type 1 and Lewis antigens) serve as putative receptors for rotavirus VP8\*, and play a role in susceptibility to infections *in vivo*. In **Paper II**, we therefore studied the effect of SNPs in the *FUT2* (rs601338) and *FUT3* genes (rs28362459, rs3894326, rs812936 and rs778986) on the serum IgG antibody titers and neutralizing antibody levels to rotavirus P[6] and P[8]. The rotavirus specific serum IgG levels and neutralizing antibody titers to the Wa strain (P[8]) of rotavirus were significantly higher in secretors (individuals with an intact *FUT2*), suggesting that secretor individuals, expressing the Lewis b antigen, are more prone to rotavirus (P[8]) infections than non-secretors.

We have recently developed an antibody-based therapy against rotavirus, which may confer safe, immediate and efficient viral neutralization and protection. Probiotic bacteria represent an attractive delivery system for antibody fragments and other proteins in the gastrointestinal tract. In **Paper III**, the combination therapy including engineered *L. rhamnosus* GG expressing IgG binding domains of protein G and HBC was shown to be more effective in reducing the prevalence, severity, and duration of diarrhea in a mouse pup model of RRV infection in comparison to HBC alone or a combination of wild-type *L. rhamnosus* GG and HBC. In **Paper IV**, we developed vectors for co-production of two VHHs: ARP1 and ARP3, by engineered *L. paracasei* BL23. Both fragments (secreted or anchored) were shown to bind to a broad range of human rotavirus serotypes *in vitro*. In **Paper V**, we have shown that the fusion of the mouse IgG1 Fc to ARP1 (Fc-ARP1) confers a markedly increased protection against rotavirus in a neonatal mouse model of rotavirus-induced diarrhea, suggesting a role for Fc-mediated neutralization of rotavirus. These antibody-based treatments could be further developed and used as an alternative or complement to current vaccines.

# LIST OF SCIENTIFIC PAPERS

- I. Mutations in toll-like receptor 3 are associated with elevated levels of rotavirus-specific IgG antibodies in IgA-deficient but not IgA-sufficient individuals.**  
Günaydın G, Nordgren J, Svensson L, Hammarström L.  
Clinical and vaccine immunology : CVI 2014; 21:298-301.
- II. The FUT2 gene determines susceptibility to rotavirus infections in a Swedish population.**  
Günaydın G\*, Nordgren J\*, Sharma S, Svensson L, Hammarström L.  
Submitted.
- III. Engineered Lactobacillus rhamnosus GG expressing IgG-binding domains of protein G: Capture of hyperimmune bovine colostrum antibodies and protection against diarrhea in a mouse pup rotavirus infection model.**  
Günaydın G, Zhang R, Hammarström L, Marcotte H.  
Vaccine 2014; 32:470-7.
- IV. Co-expression of anti-rotavirus proteins (llama VHH antibody fragments) in Lactobacillus: Development and functionality of vectors containing two expression cassettes in tandem.**  
Günaydın G, Álvarez B, Lin Y, Hammarström L, Marcotte H.  
PloS One 2014; 9(4): e96409.
- V. Fusion of mouse IgG1 Fc to llama single domain VHH (ARP1) confers increased protection against rotavirus in a neonatal mouse model of rotavirus induced diarrhea.**  
Günaydın G, Marcotte H, Hammarström L.  
Manuscript.



## **ADDITIONAL PUBLICATIONS NOT INCLUDED**

### **I. Integrative expression system for delivery of antibody fragments by lactobacilli.**

Martín MC, Pant N, Ladero V, **Günaydın G**, Andersen KK, Alvarez B, Martínez N, Alvarez MA, Hammarström L, Marcotte H.

Appl Environ Microbiol. 2011 Mar;77(6):2174-9.

### **II. The Lewis-negative phenotype is a strong restriction factor for human P[8] rotavirus infections.**

Nordgren J\*, Sharma S\*, Bucardo F\*, Nasir W, **Günaydın G**, Ouermi D, Nitiema LW, Becker-Dreps S, Simpore J, Hammarström L, Larson G and Svensson L.

Submitted.

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# LIST OF ABBREVIATIONS

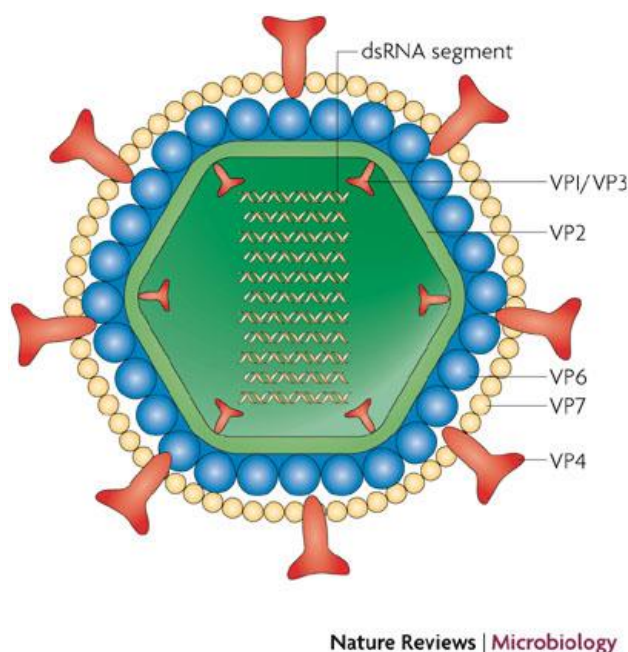
APF	Aggregation promoting factor
cDNA	Complementary DNA
CVID	Common variable immunodeficiency
DLP	Double layered rotavirus particles
dsRNA	Double stranded-ribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
FcRn	Neonatal Fc receptor
Fc $\gamma$ R	Fc gamma receptor
FUT2	$\alpha$ -1,2-fucosyltransferase encoding gene
FUT3	$\alpha$ -1,3-fucosyltransferase encoding gene
GB1-3	IgG-binding domains of protein G
HBC	Hyperimmune bovine colostrum
HBC-IgG	HBC-derived IgG antibodies
HBGA	Histo-blood group antigens
HSV	Herpes simplex virus
IEC	Intestinal epithelial cell
IFN	Interferon
Ig	Immunoglobulin
IgAD	Immunoglobulin A deficiency
Le	Lewis
MDA5	Melanoma differentiation-associated protein 5
NSP	Non-structural protein
PAMP	Pathogen-associated molecular pattern

PG3	Engineered <i>L. rhamnosus</i> GG with surface display of GB1-3
PRR	Pattern recognition receptor
RIG-1	Retinoic acid-inducible gene 1
RLR	RIG-1-like receptor
RRV	Rhesus rotavirus strain
RT-PCR	Quantitative real-time polymerase chain reaction
SCID	Severe combined immunodeficiency
Se	Secretor
SEM	Scanning electron microscopy
SNP	Single nucleotide polymorphism
TLR3	Toll-like receptor 3
TRIM21	Tripartite motif-containing protein 21
VHH	Llama variable heavy chain antibody fragment
VP	Viral structural protein
XLA	X-linked agammaglobulinemia

# 1. INTRODUCTION

## 1.1. ROTAVIRUS BIOLOGY

Rotavirus is a non-enveloped double stranded RNA (dsRNA) virus within *Reoviridae* family. Rotaviruses are classified into seven groups (A to G) on the basis of their distinct antigenic and genetic properties, with group A rotaviruses being the main cause of infection in humans (1). The mature and infectious rotavirus particle is approximately 100 nm in diameter, and composed of a three-layered icosahedral protein capsid surrounding the genome (**Figure 1**) (2, 3).

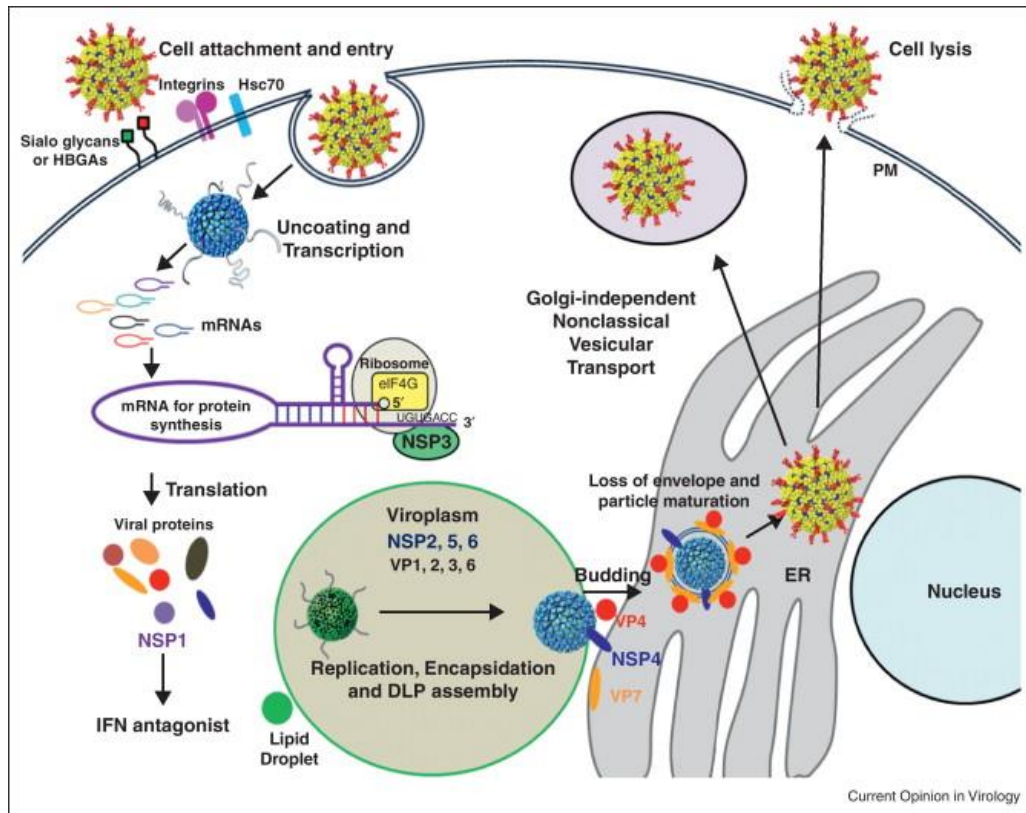


**Figure 1.** The schematic representation of a rotavirus virion (3). Reprinted with the permission from Nature Publishing Group.

The rotavirus genome contains 11 segmented dsRNA, encoding 12 viral proteins characterized in two categories: structural (VP1-4, VP6, and VP7) and non-structural (NSP1-6) proteins. The inner layer of the virion is composed of the scaffolding core protein VP2, the RNA-dependent RNA polymerase VP1, the RNA capping enzyme VP3, and genomic RNA. The intermediate layer is made

of VP6, which is the most abundant and highly conserved protein, and also the group and subgroup specific rotavirus antigen, used for classification purposes. The outer layer is composed of VP4 and VP7 proteins which elicit neutralizing antibodies and form the basis of the current dual classification of group A rotavirus into G- (glycoprotein VP7) and P- (protease-sensitive protein VP4) types (**Figure 1**).

The outermost layer of rotavirus virion is lost during the cell entry, yielding transcriptionally active double layered rotavirus particles (DLPs) (**Figure 2**) (4). DLPs are responsible for transcription of viral mRNAs. The plus strand RNA is extruded from DLPs through channel and translated into viral proteins. The synthesized rotavirus NSP proteins mediate replication of rotavirus (**Figure 2**).



**Figure 2.** The rotavirus replication cycle (4). Reprinted with the permission from Elsevier.



The NSP1 protein is engaged in inhibition of IFN- $\alpha$  responses, NSP2 is required for dsRNA synthesis, and NSP3 is essential for translational regulation and inhibition of host protein synthesis. The viral enterotoxin NSP4 increases the concentration of  $\text{Ca}^{2+}$ , which disrupts the cytoskeleton of microvilli and the cellular homeostasis of the host. NSP4 is the major contributing factor to electrolyte and fluid malabsorption causing diarrhea. In addition, NSP5 interacts with NSP2 to form cytoplasmic structures known as viroplasms, inside of which RNA replication and morphogenesis of new viral particles take place. NSP6 interacts with NSP5 in the viroplasms, but its function is unknown, being not coded by all rotavirus strains (3, 4).

## **1.2. PATHOGENESIS OF ROTAVIRUS INFECTIONS AND EPIDEMIOLOGY STUDIES**

Rotavirus is the most crucial pediatric pathogen, infecting infants and children younger than 5 years of age, and is associated to several clinical symptoms including acute diarrhea, nausea, abdominal pain, fever, and in some cases, vomiting.

The virus infects the mature enterocytes of the small intestine (2). The apical villi undergo atrophy and crypt hyperplasia, leading to nutrient malabsorption and increased secretion, hence diarrhea. These series of events lead to intestinal tract infection, also referred to as acute gastroenteritis.

The rotavirus infections cause approximately 500 000 annual deaths worldwide, 85 % of which occur in low and middle income countries. Based on a recent report of World Health Organization (WHO), rotavirus induced acute gastroenteritis caused mortality constitutes 5 % of all child deaths (5). In addition, an annual approximate of 2 million hospitalizations and 25 million clinic visits, caused by rotavirus induced gastroenteritis, has been correlated to significant economic burden worldwide, especially in the developing world (6).

### 1.3. DISTRIBUTION OF ROTAVIRUS GENOTYPES

Group A rotavirus strains are classified in 23 G types and 31 P types known so far. The global distribution of common rotavirus G/P type combinations is as follows: G1 P[8] (31 %), G2 P[4] (19 %), G3 P[8] (10 %), G9 P[8] (6 %) and G4 P[8] (4 %) (7). These five most common genotypes are detected in more than 85 % of infected individuals both in Europe and America, however they account for only 40 % of circulating genotypes in Africa. The combinations other than aforementioned genotypes are called “uncommon”, and account for 20 % of all genotypes in the world. The frequency of uncommon rotavirus genotypes circulating in Europe (9 %) and America (8 %) is lower as compared to in Africa (35 %) (7). Remarkably, G1 P[8], the most prevalent rotavirus genotype circulating in Europe (33 %) and America (43 %), constitutes only 14 % of all circulating rotavirus genotypes in Africa (7). The diversity among strains along with geographical and temporal variations, and emergence of new strains potentially poses a challenge in the development of vaccine and other forms of immune therapy. In addition, current monovalent and pentavalent vaccines include only the P[8] genotype. Host genetic factors influence rotavirus P[8]-specific responses, which may be associated to reduced efficacy with currently licensed vaccines in the developing world as compared to developed countries.

### 1.4. HOST GENETIC FACTORS IN ROTAVIRUS INFECTIONS

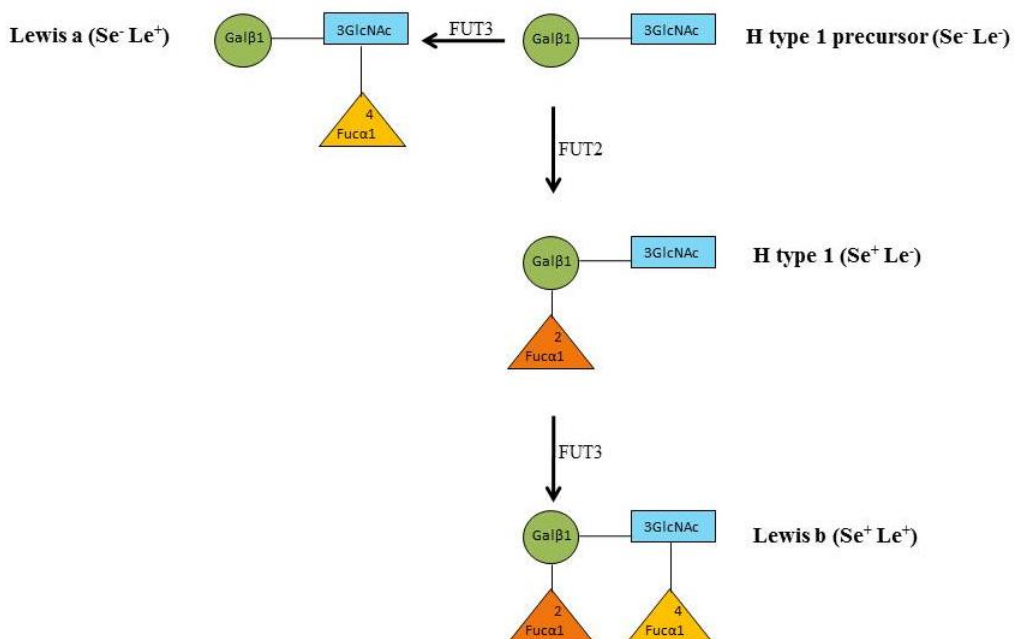
#### 1.4.1. Cell Surface Receptors

The outer capsid protein VP4 mediates rotavirus attachment and viral entry to mature enterocytes by its trypsin activated glycan binding domain, also referred to as VP8\* (**Figure 2**) (8). Earlier *in vitro* studies showed that the infection of the host cell by some animal rotavirus strains is dependent on sialic acid residues (9). Subsequent studies showed that the requirement of sialic acid is associated with the P genotypes but independent of the host origin of the strains (10). Animal and human rotaviruses with P[1], P[2], P[3], and P[7] genotypes recognize terminal sialic acid residues of carbohydrates on the host cell surface

(referred to as sialidase-sensitive) (10, 11), while many other animal and human rotaviruses (with common genotypes of P[4], P[6], and P[8]) are sialidase-insensitive. The sialidase-insensitive strains might recognize internal sialic acid residues (12), but the role of subterminal sialic acid in infection with rotaviruses has not been demonstrated. The lack of a defined receptor for the sialidase-insensitive rotaviruses has prompted further studies.

The type 1 chain histo-blood group antigens (HBGAs) are expressed in several cell types including intestinal epithelial cells, and found in biological fluids such as blood, saliva, and milk (13). They have been previously identified as cell attachment and susceptibility factors for norovirus (14) and *Helicobacter pylori* (15) infections. A recent key discovery by Hu *et al.* shows that A-type HBGAs bind to human rotavirus VP8\*, and this binding site is similar to the terminal sialic acid attachment domain on animal rotavirus VP8\* (16). In addition, recent *in vitro* studies demonstrate that human rotavirus VP8\* recognizes HBGAs in a P-genotype specific manner (10, 17), suggesting a genetic predisposition for susceptibility to distinct rotavirus strains.

The  $\alpha$ -1,2-fucosyltransferase enzyme, encoded by secretor (*FUT2*) gene, converts the type 1 chain precursor to H type 1 antigen (**Figure 3**). In addition,  $\alpha$ -1,3-fucosyltransferase enzyme, encoded by Lewis (*FUT3*) gene, converts H type 1 antigen to Lewis b ( $Le^b$ ), and type 1 chain precursor to Lewis a ( $Le^a$ ). The single nucleotide polymorphisms (SNPs) in *FUT2* and *FUT3* genes are thus associated with distinct expression patterns of HBGAs, determining genetic variability between populations. While the prevalence of a *FUT2* SNP (rs601338), causing a non-secretor phenotype, is approximately 30 % in the Caucasian population (18, 19), it is presented in approximately 65 % in south Africans (20). In addition, the four SNPs of the *FUT3* gene (rs28362459, rs3894326, rs812936 and rs778986) have previously been shown to be associated with a Lewis negative phenotype (21), being 4-6 % in Caucasian vs. 22- 32 % in African populations (21-23).

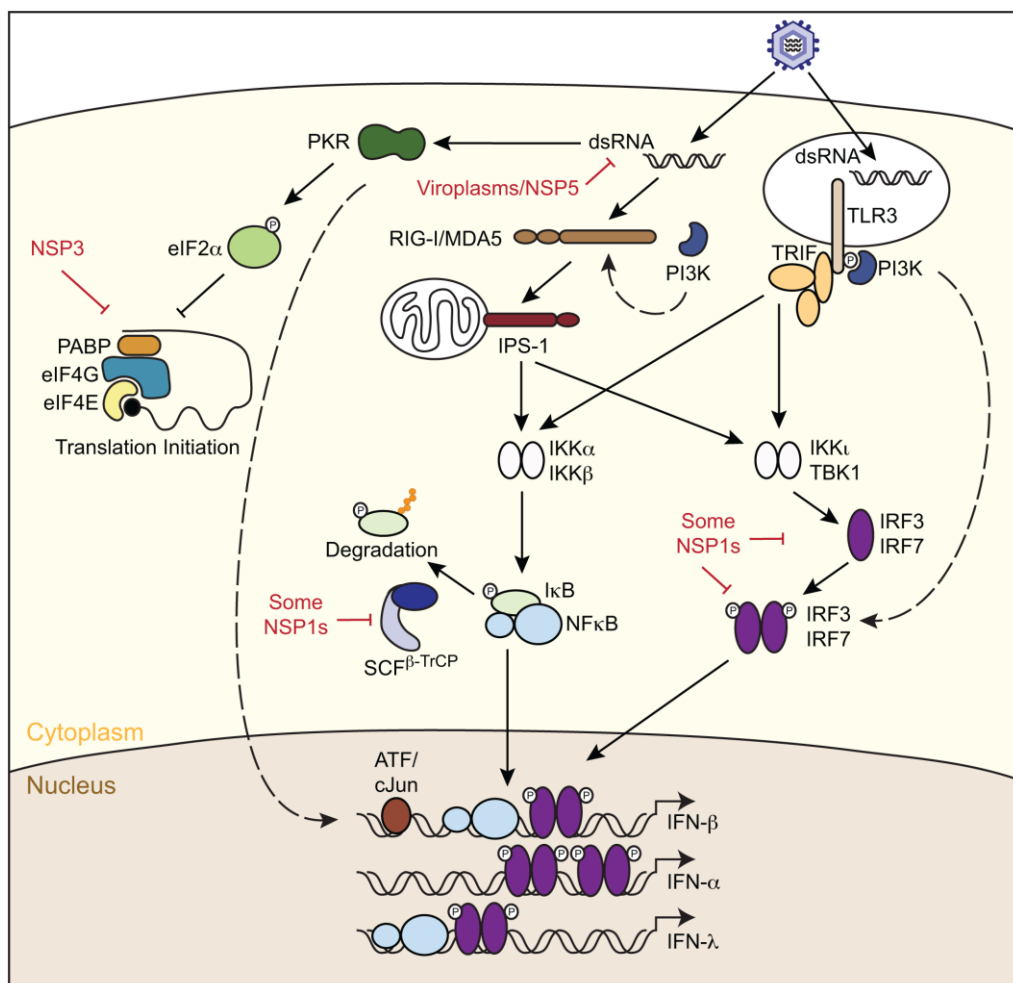


**Figure 3.** The synthesis of type 1 HBGAs mediated by the *FUT2* and *FUT3* enzymes rendering Secretor (*Se*) and Lewis (*Le*) phenotypes (Paper II).

The rotavirus strains of P[4] and P[8] genotypes can bind to Lewis b and H type 1 blood group antigens, while P[6] rotavirus strains can only bind to the H type 1 antigen (10). The secretor (*FUT2*) gene, mediating the expression of H type 1 and Lewis b antigens, have been proposed as susceptibility factors to rotavirus (of P[8] genotype) infections in a recent study *in vivo* (24). The resistance to P[8] rotavirus infections through non-secretor phenotype might clarify the reduction in efficacy of current P[8]-based vaccines used in the developing world including sub-Saharan Africa.

#### 1.4.2. Intracellular Receptors

Once rotavirus enters into the small intestinal cell, the viral transcription machinery is consecutively activated to produce viral components including viral nucleic acids, of which intracellular receptors sense and induce antiviral innate immune responses (**Figure 4**).



**Figure 4.** Rotavirus interactions with innate signaling pathways (25). Open access for reprinting, PLOS Publishing Group.

Toll-like receptors (TLRs), belonging to the pattern recognition receptor (PRR) family, are expressed in intestinal epithelial cells (IECs) in a polarized pattern (26). Each member of the TLR family is specialized in recognition of various ligands with distinct pathogen-associated molecular patterns (PAMPs) (27). While some TLRs are present at the cell surface, others including TLR3, sensing dsRNA, are located inside the cell at the endosomes. TLR3 activation further engages TRIF adaptor molecules in the stimulation of nuclear mediators such as NF-κβ, IRF3, CREB and AP1 to induce type I (IFN-α/β) and type III (IFN-λ)

IFNs, and other cytokines (TNF, IL-1 $\beta$ ) or chemokine (IL-8) expression (27-30). In addition, melanoma differentiation-associated protein 5 (MDA5) and retinoic acid-inducible gene 1 (RIG-1), being members of the RIG-1-like receptor (RLR) family within PRR family, are also involved in intracellular rotavirus sensing. Even though cytosolic helicases MDA5 and RIG-1 distinctly recognize viral dsRNAs in a length-dependent manner (31), they might also work in combination to induce protective IFN responses in rotavirus infected IECs (32).

Over 100 single nucleotide polymorphisms (SNPs) have been identified in the human *TLR3* gene, some of which are predicted to be damaging, and associated with susceptibility to selected viral infections. According to PolyPhen/SIFT analysis, two *TLR3* gene polymorphisms (rs3775291 and rs5743305) cause nonfunctional and reduced levels of TLR3, respectively. The L412F (rs3775291) SNP is associated with susceptibility to herpes simplex virus (HSV-type 1) (33), tick-borne encephalitis virus (TBEV) (34) and measles virus (35) infections in humans. Additionally, the rs5743305 promoter variant shows lower measles-specific antibody titers in response to vaccination (35).

A recent study by Pott *et al.* shows that the age-dependent predisposition to rotavirus infections might be due to the low expression of *TLR3* in young humans and neonatal mice (36). Additionally, adult mice with a targeted inactivation in the *TLR3* gene show increased level of rotaviral shedding (36). The absence of intact TLR3 signaling might cause viral pathogenesis and development of rotavirus gastroenteritis.

## **1.5. IMMUNITY TO ROTAVIRUS INFECTIONS**

### **1.5.1. Humoral Immunity**

Early studies on natural rotavirus infection in healthy children suggest that serum anti-rotavirus antibodies, especially immunoglobulin A (IgA), correlates well with protection from rotavirus infections, and both symptomatic and asymptomatic infections result in similar protection levels (37). Mucosal immunity also seems to play a crucial role as the presence of high levels of anti-rotavirus IgA antibodies in fecal samples has been strongly associated with

protection (38, 39). The total serum anti-rotavirus IgA level, assessed shortly after infection, generally reflects intestinal IgA levels and appears to be the best marker of protection (40). However, gut immunity is of short term and difficult to measure. Remarkably, neutralizing secretory IgA antibody confers protection against rotavirus in an infant mouse model (41, 42). The viral neutralization mechanisms of secretory IgA might involve immune exclusion and intracellular viral inactivation during transcytosis of IgA in small intestine (41, 43). Even though IgA is defined as the major contributor of protection, elevated titers of serum anti-rotavirus IgG are detected in individuals with IgA deficiency (IgAD) (44, 45), with serum IgA levels lower than 0.07 grams per liter, as compared to healthy individuals, suggesting a compensatory protective role of IgG antibodies in rotavirus infections (44, 46).

Although VP6 is an immunodominant antigen in the antibody response to human rotavirus infection (47), rotavirus outer capsid proteins VP4 and VP7 are crucial in eliciting neutralizing antibodies (48). However, some antibodies against VP6 (49, 50), and enterotoxin NSP4 (51, 52) might also confer protection against rotavirus.

### **1.5.2. Cell-mediated Immunity**

The contribution of cell-mediated immune response for protection against rotavirus infections is relatively unknown in human, but is most likely essential in resolution of the infection. The counts of rotavirus specific interferon gamma-secreting CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes are somewhat low in children with acute rotavirus induced diarrhea (53). In addition, rotavirus-specific T helper (T<sub>H</sub>) cells are detected in blood samples from infants after primary symptomatic rotavirus infection (54). Dendritic cells induce production of rotavirus specific T<sub>H</sub>1 cells, when infected with rotavirus *in vitro* (55). Furthermore, natural killer cells were found to be up-regulated via IL-15 induction after exposure to different rotavirus strains *in vitro* (51).

In mice, the CD4<sup>+</sup> T lymphocytes are essential for the development of the majority of RV-specific intestinal IgA (56). Furthermore, murine rotavirus-

specific CD8<sup>+</sup> T lymphocytes are engaged in the gradual resolution of primary rotavirus infection and induce partial protection against reinfection (57, 58).

## **1.6. HUMAN ROTAVIRUS VACCINES**

The first rotavirus vaccine, Rotashield<sup>TM</sup> (Wyeth-Lederle Vaccines), a tetravalent rhesus-human reassortant vaccine, was withdrawn from the market due to gut intussusception cases in 1999. Two new oral vaccines were subsequently developed and licensed in 2006. Rotateq<sup>TM</sup> (Merck) is a live pentavalent bovine-human reassortant vaccine, consisting of G1, G2, G3, G4 and P1A[8] types, as the representatives of co-circulating common wild-type rotavirus strains (59). Multivalent vaccines aim to induce a stronger and cross-neutralizing antibody response. However, Rotatix<sup>TM</sup> (GlaxoSmithKline Biologicals), obtained from a clinical human isolate, is an attenuated monovalent (G1 P1A[8]) vaccine (59).

Rotatix<sup>TM</sup> and Rotateq<sup>TM</sup> are included in national vaccination programs of several countries, and have been shown to be safe within the restricted age limitation in use (between 6-26 weeks of age). The Phase III trials report high efficiency (>85 %) in reducing severe rotavirus-induced diarrhea in developed countries, and a less pronounced decline in rotavirus associated mortality rates in developing countries (60-62). The vaccine efficacy is reduced in some areas especially in South Africa and Asia, where rotavirus infection is the single most deadly pathogen among children. Rotavirus vaccination elicits both homotypic and heterotypic immunity, and the protection is correlated with rotavirus specific serum IgA and IgG antibodies (51, 63).

Certain risk groups should avoid vaccination, including older infants and infants with immunodeficiency disorders (64-68), as indicated in the contraindication reports from the two aforementioned companies. The safety and immunogenicity of vaccines on HIV infected immunosuppressed children should also be studied extensively (69).



## 1.7. PASSIVE ANTIBODY-BASED THERAPIES

Passive immunization might represent an alternative treatment option for children with impaired immunity due to absence of protective antibodies (children with IgAD and CVID), with co-infections (HIV, enteric pathogens, etc.), malnutrition, genetic susceptibility factors (distinct HBGAs), and genetic defects (SNPs in genes affecting innate immune responses, i.e. TLR3, MDA5/RIG-1).

Antibody-based therapies are successfully used for reducing or curing rotavirus induced diarrhea in human and animal models. Neutralizing monoclonal IgA antibodies against the VP4 protein, administered in a mouse hybridoma backpack tumor model for secretion onto mucosal surfaces via the normal epithelial transport pathway, elicit prophylactic protection in mice orally challenged with rotavirus (41, 50). Remarkably, IgA antibodies against double layer protein VP6, non-neutralizing in classical *in vitro* assays using non-polarized cells, mediate protection in murine hybridoma backpack tumor model, but not in an orally fed mouse model (50). The monoclonal antibodies, when applied to the basolateral pole of polarized Caco-2 intestinal cells, can reduce viral replication, and suppress the loss of barrier function which is mediated by apical exposure of the cell monolayer to rotavirus (43). This suggests that the antiviral activity of antibodies depends on activation of intracellular antiviral mechanisms during transcytosis.

Passively administered antibodies might use distinct mechanisms of neutralization. Monoclonal antibodies against VP4 and VP7 have been shown to prevent rotavirus attachment (48) and viral decapsidation (70), respectively. Antibodies against VP6 antibodies were shown to inhibit genome transcription and viral transcription *in vitro* (71).

In addition to their antigen blocking activity, antibodies, depending on their origin, are able to induce or suppress immune responses in cells through Fc/Fc receptor interactions, by binding to Fc gamma receptor (Fc $\gamma$ R), neonatal Fc receptor (FcRn) or cytosolic Fc receptor tripartite-motif containing protein 21

(TRIM21). Based on the level of infection, one or more Fc receptor type(s) may be activated, causing different intracellular effector functions.

Several studies have shown that oral administration of hyperimmune polyclonal bovine colostrum or hyperimmunized chicken egg yolk immunoglobulin is protective against rotavirus in animal models and humans (72-75).

Even though passive transfer of immunoglobulins elicits safe immediate immune protection even in immunodeficient individuals, antibody production and purification procedures can be costly, and new methods for production and delivery of antibodies are in need.

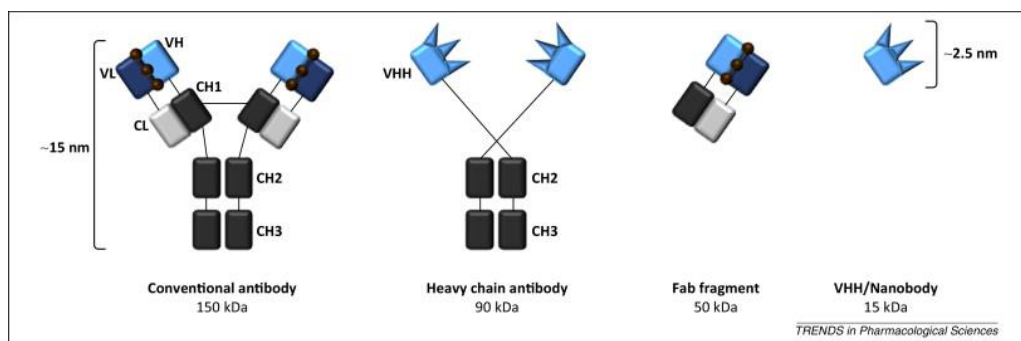
### **1.7.1. Bovine Colostrum Antibodies**

The cow can produce approximately 1-1.5 kg of immunoglobulins in the first few days after calving, making it an attractive platform for large-scale antibody production. Bovine colostrum-based immune milk derived antibodies, mainly IgG, have been used against gastrointestinal tract infections, including cryptosporidiosis, shigellosis, rotavirus, enterotoxigenic *E. coli*, and *C. difficile* infections (76, 77).

Hyperimmune bovine colostrum (HBC) fed to rotavirus-infected calves (78) and infant mice (79-81) conferred a high level of protection, in a dose-dependent manner. Orally administered HBC antibodies are successfully used for treatment of rotavirus in children with rotavirus-induced diarrhea (82, 83). Remarkably, the most abundant immunoglobulin, colostrum IgG<sub>1</sub>, when given orally to mice, can resist proteolytic enzymes in the stomach (84), and retain high and stable activity in the small intestine for approximately 3.5 hours following administration (85). In humans, 50% of administered bovine antibodies resist digestion in the human upper gastrointestinal tract (86), suggesting that HBC antibodies can potentially be used as alternative, or complementary, treatment to rotavirus infections, especially in rotavirus vaccine risk groups.

### 1.7.2. *Camelidae* Family Heavy Chain Antibodies

The novel discovery (1989) of *Camelidae* family heavy chain-only single-domain antibodies (referred to as Nanobodies® or VHHs), started a new era for anti-viral therapy against rotavirus (79, 87-89), influenza-A (90, 91), poliovirus (92), HIV-1 (92, 93) and HBV (94). The VHH fragments are the smallest (12-15 kDa in size) naturally available antigen binding domain known so far (95), with high acid and heat resistance, providing stability and functionality *in vivo*, and are able to reach cryptic epitopes that are inaccessible to conventional antibodies (Figure 5) (96). These properties make them suitable for therapy at mucosal sites such as the gastrointestinal tract where the acidic pH can limit the functionality of conventional antibodies.



**Figure 5.** Structural features of different antibodies (97). Reprinted with the permission from Elsevier.

Rotavirus specific VHH antibody fragments (named ARP1 and ARP3), were isolated from a llama VHH phage display library after immunizing the llama with RRV. ARP1 and ARP3 are the two VHHs that have been extensively studied in our group for therapy against rotavirus infections and were used successfully against rotavirus infections in an infant mouse model (88). Both fragments recognize polymeric VP6, which could be a conformational epitope, and are widely cross-reactive to different simian and human rotavirus strains, including the most prevalent ones (49). In a randomized placebo-controlled trial, ARP1 has been shown to be safe, and to reduce stool outcome in infants with

acute rotavirus diarrhea (98). Functional ARP1 fragments can be produced in yeast (88), *Lactobacillus* (87), and rice as a food-grade product (99).

Multimerization of VHH fragments with different specificities can potentially increase cross-reaction capacity to several co-circulating genotypes of rotavirus, and reduce the appearance of escape mutants. An improved effect of combination of two or more monoclonal antibodies on virus neutralization has previously been demonstrated (100). In addition, fusion of 2-3 VHH fragments against viruses including respiratory syncytial virus (RSV), rabies and influenza H5N1 improves neutralization efficacy of single VHH fragments by 75-4000 fold, by increasing avidity, mediating agglutination of viruses, and broadening neutralization of more serotypes, and some multimeric VHHs against RSV can reduce formation of viral escape mutants (90).

## **1.8. LACTOBACILLUS-BASED THERAPIES: AN ANTIBODY DELIVERY SYSTEM**

Lactobacilli are Gram-positive lactic acid bacteria, which form a part of intestinal microbiota. From early 20<sup>th</sup> century till date, many studies have shown their safety in the food industry, so they are referred to as “Generally Regarded as Safe (GRAS) microorganisms”. According to the report of FAO/WHO, probiotics are defined by: “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (101). Several studies show host health benefits associated to use of certain *Lactobacillus* strains. Lactobacilli produce bacteriocins, short chain fatty acids, enzymes and vitamins which compete with pathogens for attachment to epithelial receptors, and induce anti-inflammatory and immunostimulatory responses (102). The best lactobacilli colonizers in gastrointestinal tract are *L. gasseri*, *L. reuteri*, *L. casei* and *L. salivarius* (103). *L. rhamnosus* GG, a well-studied probiotic strain in gastrointestinal tract infections, reduces duration of infantile diarrhea caused by rotavirus infections (79, 104, 105). The probiotic activity of *L. rhamnosus* GG is characterized by their colonization of the gastrointestinal tract, their ability to strengthen the intestinal barrier and to trigger innate and adaptive immune responses (106, 107).

Engineered lactobacilli for *in situ* delivery of antibodies or antibody fragments have been used successfully in treatment of rotavirus diarrhea (87, 108). The genetic modifications of *Lactobacillus*, in our studies, are based on expression of vectors comprising the promoter and signal peptide of the *apf* gene of *L. crispatus* M247, the gene encoding the antibody fragment or protein of interest, the gene encoding a tag for detection, and a sequence encoding the proteinase P surface protein (PrtP) of *L. paracasei* BL23 or *L. rhamnosus* GG for covalent anchoring on the cell surface. While lactobacilli producing surface-anchored VHH fragment reduced rotavirus diarrhea in a neonatal mouse model, the same antibody fragment was not protective when secreted by the same strain of lactobacilli due to a suboptimal production rate. Since the anchored antibody fragments are saturated on the surface of lactobacilli, they might inhibit rotavirus binding to the cell receptor, prior to cell infection.

Multimeric display of anti-HIV antibodies on *Caulobacter* displaying protein G, markedly enhanced virus inhibition due to an increased avidity to HIV, as compared to soluble antibodies alone (109). In addition, surface anchored VHH dimer (ARP3-ARP1) produced by *Lactobacillus*, improves the therapeutic effect as compared to monovalent fragments and reduces rotavirus diarrhea in an infant mouse model (87).

## **1.9. ANIMAL MODELS OF ROTAVIRUS INFECTION**

Several animal models including mouse, rat, rabbit, piglet, calf, lamb and baboon models are used in rotavirus infection and natural protection studies (51). However, the mouse and piglet models have been instrumental for infection, natural immunity, protection, and vaccine immunity studies.

### **1.9.1. Mouse Model**

Mice at any age can be infected with rotavirus, and shed rotavirus particles in their stool. Although adult mice are asymptomatic, not showing clinical symptoms of rotavirus infection (infection-only model), young mice (less than 2 weeks of age) provide both an infection and disease model (110). Both homologous and heterologous rotavirus strains, isolated from the same and

different species, respectively, are used for infection in mouse models. The lower replication rate of heterologous strains and absence of their horizontal spread to other uninoculated mice, have made them convenient models in several studies (111-113). In addition, the use of the simian rotavirus strain in mouse pups leads to physiopathological symptoms in pups identical to those produced by the corresponding murine strain (88). It has been shown that intestinal and serum anti-rotavirus IgA levels are correlated to protection in mice (51). Furthermore, administration of rotavirus specific antibodies or *Lactobacillus* expressing VHH antibody fragments neutralizes rotavirus efficiently, in a dose-dependent manner (79, 108).

## 2. AIMS

### 2.1. GENERAL AIMS OF THE THESIS

The main objectives of the thesis were to investigate host genetic factors leading to differential susceptibility to rotavirus infections and to develop antibody-based oral therapy against rotavirus infections.

### 2.2. SPECIFIC AIMS

**Paper I.** To unravel the role of TLR3 in rotavirus-specific IgG antibody responses in Swedish individuals with IgA deficiency as compared to Swedish healthy blood donors.

**Paper II.** To investigate the involvement of human blood group antigens in rotavirus-specific IgG and neutralizing antibody responses in Swedish individuals with IgA deficiency and Swedish healthy blood donors.

**Paper III.** To develop a combination therapy, consisting of engineered *L. rhamnosus* GG expressing surface anchored IgG binding domains of protein G, and soluble anti-rotavirus HBC antibodies.

**Paper IV.** To generate *L. paracasei* BL23 expressing two rotavirus-specific llama VHH fragments (ARP1 and ARP3) in secreted and cell wall-anchored forms.

**Paper V.** To evaluate the role of Fc-mediated anti-rotavirus activity, by fusing ARP1 to the Fc fragment of mouse IgG1 (Fc-ARP1).

## 3. MATERIALS AND METHODS

### 3.1. SERUM SAMPLES

Serum samples from Swedish IgA deficient and Swedish healthy blood donors were used in *Paper I* and *Paper II*. The regional ethical review board in Stockholm, Sweden approved these aforementioned studies with the following permits: Dnr 2011/69-31/3 and Dnr 2013/1176-31/1.

In *Paper I*, serum samples from 783 Swedish IgA-deficient individuals and 1009 anonymous healthy blood donors were collected for the *TLR3* genotyping analysis. 180 IgA-deficient and 198 healthy individuals were included in the rotavirus-specific IgG antibody titer measurements.

In *Paper II*, serum samples from 767 Swedish IgA-deficient individuals and 1008 anonymous healthy blood donors were collected for the *FUT2* and *FUT3* genotyping analysis. Among all individuals, the number of individuals included in rotavirus-specific IgG antibody titer assessment was narrowed down to 378 individuals (180 IgA-deficient and 198 healthy individuals). In addition, for evaluation of serum neutralizing antibody responses, the serum samples from 41 IgA-deficient and 48 healthy individuals were used in this study.

### 3.2. GENOTYPING

SNP genotyping of the *TLR3* (rs3775291 and rs5743305) in *Paper I*, and *FUT2* (rs601338) and *FUT3* (rs28362459, rs3894326, rs812936 and rs778986) genes in *Paper II* were performed at the Mutation Analysis Facility (MAF) at Karolinska Institutet, Stockholm, Sweden. SEQUENOM platform was used based on a matrix assisted laser desorption ionization-time of flight (MALDI-TOF) analysis.

### 3.3. NEUTRALIZATION ASSAY

The neutralization of rotavirus strains Wa and ST3 by serum samples was in MA104 cells by the immunoperoxidase assay in *Paper II*. The serum dilution,



leading to higher than 60 % reduction in the number of stained infected cells was considered to be the neutralizing antibody titer.

### **3.4. BACTERIAL STRAINS**

*L. rhamnosus* GG, used in *Paper III*, was provided by Prof. Marika Mikelsaar, University of Tartu, Estonia. *L. paracasei* BL23 (previously referred to as *L. casei* 393, or pLZ15<sup>+</sup>), used in *Paper IV*, was obtained from Dr. Peter Pouwels, TNO Institute, the Netherlands. *Lactobacillus* strains were grown in MRS broth (Difco, Sparks, MD) in standing aerobiosis conditions at 37°C. MRS medium was supplemented with 5 microgram per milliliter of erythromycin for *Lactobacillus* transformants.

### **3.5. ROTAVIRUS STRAINS**

The simian rotavirus strain RRV was used in all studies. In *Paper I* and *II*, it was used to evaluate the rotavirus-specific IgG responses. In *Paper III* and *V*, RRV strain was used for viral challenge in neonatal mouse pups. Furthermore, RRV was used in analysis of virus binding in *Paper III-V*. Another simian rotavirus strain, SA11, was used in virus binding assay in *Paper IV*. Human rotavirus strains Wa and ST3 were used for neutralization studies in *Paper II*. In addition other human rotavirus strains including Wa, ST3, 69M, Va70, F45 and DS1 were used in *Paper IV*.

The rotavirus strains were grown, harvested from mammalian cells, and the viral titers were calculated as mentioned previously (114, 115). The RRV strain was kindly provided by Ass. Prof. Kari Johansen, Department of Microbiology, Tumor and Cell Biology, Karolinska Institute, Stockholm, Sweden. Additionally, Wa, ST3, 69M, Va70, F45, DS1 and SA11 were kindly supplied by Prof. Miren Iturriza-Gómara, Enteric Virus Unit, Centre for Infections, Health Protection Agency, London, UK.

### **3.6. GENERATION OF ARPS: ARP1 AND ARP3**

Immunization of llamas with RRV strain MMU18006, P5B, G3, followed by selection of ARP1 and ARP3 has previously been described (88). ARP1 was previously referred to as 2B10 (88) or VHH1 (108). ARP1 and ARP3 produced in *Saccharomyces cerevisiae*, were purified using ion exchange chromatography by BAC BV (The Netherlands). The purity was found to be higher than 95 %. The ARP1 was used in *Paper IV* and *V*, and ARP3 in *Paper IV*.

### **3.7. HYPERIMMUNE BOVINE COLOSTRUM ANTIBODY PREPARATION**

The HBC, used in *Paper III*, was produced by vaccination of pregnant cows in a Swiss dairy farm with human strains of rotavirus (Wa, RV3, RV5 and ST3) representing the serotypes of 1 to 4 (116). The freeze-dried HBC concentrate is composed of 28,1% IgG, 6,1% IgA and 2,2% IgM antibodies. The HBC preparation was kindly provided by Dr. Harald Brüssow, Nestlé Research Center, Nutrition and Health Department/Food and Health Microbiology, Lausanne, Switzerland.

### **3.8. CLONING IN *LACTOBACILLUS***

In *Paper III*, the IgG binding domains of protein G of *Streptococcus* sp. Group G was cloned in pAF900 plasmid and expressed on the surface of *L. rhamnosus* GG.

In *Paper IV*, three different co- expression cassettes were constructed where the two ARP fragments (ARP1 and ARP3) can both be secreted in the medium, one secreted and the other anchored on the cell surface, or both covalently anchored on the cell surface. The vectors were transformed into *L. paracasei* BL23.

### **3.9. EXPRESSION AND PURIFICATION OF F<sub>C</sub>-ARP1 AND F<sub>C</sub>-ARP1<sub>N434D</sub>**

In *Paper V*, the gene encoding the Fc fragment of mouse IgG1 (hinge region and C<sub>H</sub>2-C<sub>H</sub>3 domains) was fused to the gene encoding ARP1. Site-directed mutagenesis was used as a service of GenScript, to generate a single-point

mutation in the Fc-ARP1 gene replacing the asparagine corresponding to the position 434 of the original heavy chain sequence with aspartic acid (N434D). This mutation was previously shown to markedly impair neutralization of adenovirus *in vitro*, by inhibiting IgG Fc binding to TRIM21. Fc-ARP1 and Fc-ARP1<sub>N434D</sub> were transiently expressed in HEK 293-6E cells and purified (GenScript (Piscataway, NJ)).

### **3.10. ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)**

This method was used for detection of RRV-specific antibody responses in *Paper I* and *II*, for quantification of HBC IgG antibodies in *Paper III*, for evaluation of binding of antibody and antibody fragments to rotavirus in *Paper IV* and *V*.

### **3.11. EXPRESSION ANALYSIS**

The production of surface-anchored IgG binding domains of protein G (GB1-3) by *L. rhamnosus* GG in *Paper III*, and co-expressed ARP1 and ARP3 fragments in secreted and surface-anchored forms by *L. paracasei* BL23 in *Paper IV* were determined by Western blot. In *Paper V*, llama conventional antibody-like ARP1-Fc antibody and its derivatives were detected both in Western blot and SDS-PAGE gel stained with Coomassie brilliant blue.

### **3.12. SCANNING ELECTRON MICROSCOPY (SEM)**

The *L. rhamnosus* GG cells were incubated with PBS only (-ive control) or a mix of RRV ( $10^8$  ffu/ml) and anti-rotavirus HBC (10 µg/ml) in *Paper III*. The cells were fixed with 0.1 ml 2 % glutaraldehyde, and the samples were consecutively analyzed by SEM in the electron microscopy unit (EMil) at the Karolinska University Hospital Huddinge, Sweden.

### **3.13. FLOW CYTOMETRY ANALYSIS**

Flow cytometry analysis was used in *Paper III* and *IV* for detection of surface-displayed antibody fragments and antibodies on the surface of lactobacilli, and to determine the binding of lactobacilli displaying antibody and antibody fragments

to rotavirus. Following incubations with antibodies and/or rotavirus, engineered *Lactobacillus* cells were pelleted down and washed prior to fixation with 0.5 % paraformaldehyde (PFA). Cell staining data were acquired by FACS Calibur machine (Becton, Dickinson and Company), and analyzed in FlowJo software (TreeStar, Ashland, OR).

### **3.14. NEONATAL MOUSE MODEL OF ROTAVIRUS DIARRHEA**

All the animal experiments, in *Paper III* and *V*, were approved by the local ethical committee of the Karolinska Institutet at Karolinska University Hospital Huddinge, Sweden. An infant mouse model of heterologous infection with rhesus rotavirus (RRV) was used to evaluate the effect of distinct prophylactic treatments in protection against rotavirus induced diarrhea. Fourteen day pregnant, rotavirus-free certified, wild-type BALB/c mice were purchased from Charles River Lab, Germany. The experiments were started with 4 day old pups that were randomly distributed in groups of 7–8 pups per dam. Antibodies or engineered lactobacilli were administered orally, once daily in 10 µl volume, starting from one day before the infection (day -1), until the last day of the experiment (day 4, in *Paper III*, and day 5, in *Paper V*). On the infection day (day 0), each mouse pup was infected once with RRV in a 10 µl volume, two hours after the treatment with antibodies or lactobacilli.

Diarrhea was recorded from the start until the last day of the experiment. Severity of diarrhea was scored based on the consistency of stool outcome. On the last day of the experiment, sections of small intestine were collected from euthanized mouse pups for subsequent real-time PCR analysis.

### **3.15. QUANTITATIVE REAL-TIME PCR (RT-PCR)**

All small intestinal samples were homogenized using the TissueLyser MM301 system (Retsch GmbH, Haan, Germany). RNeasy RNA isolation kit (Qiagen GmbH) was used for total RNA isolation. In the process of RNA purification, on-column DNA digestion was performed using a RNase-free DNase set (Qiagen GmbH). cDNA was synthesized from 1 µg of total RNA using a first-

strand cDNA synthesis kit (GE Healthcare) based on manufacturers` protocol. The SyBr Green detection system was used in RT-PCR analysis.

The RRV VP7 viral mRNA was amplified to evaluate the viral load in *Paper III* and *V*, using a pair of specific primers: 5`-GCAATGTCCAAAAGATCACG-3` and 5`-GGTCACATCATACATTTCTATCCAA-3`.

Both *IFN-β* and *TRIM21* gene expression analysis were performed in *Paper V*. For quantification of interferon beta (IFN-β) expression levels, the following primer pairs were used: 5`-AAGAGTTACACTGCCTTTGCCATC-3` and 5`-CACTGTCTGCTGGTGGAGTTCATC-3`. The assessment of the *TRIM21* gene expression was performed using the primer pairs: 5`-CTGCAGGAGCTGATCTCAG-3` and 5`-TGTCCTCAGCATCTTCTTC-3`.

## 4. RESULTS

### 4.1. PAPER I

Individuals with IgA deficiency showed significantly elevated serum levels of rotavirus-specific IgG, suggesting a compensatory role of IgG in rotavirus infection and a protraction of rotaviral persistence/shedding after infection, although the disease is ultimately resolved. Upon the recent findings of low *TLR3* expression being involved in susceptibility to rotavirus infections (36), we investigated rotavirus-specific IgG antibody responses from individuals (IgA competent or deficient) with two well-characterized and highly damaging *TLR3* single nucleotide polymorphisms (rs3775291 and rs5743305). We concluded that these two polymorphisms were associated with elevated IgG titers in IgA deficient, but not in IgA competent individuals.

### 4.2. PAPER II

The histo-blood group antigens were previously shown to serve as receptors for rotavirus VP8 on the host cells (10, 24). In *Paper II*, we have studied the effect of inactivating mutations in the *FUT2* (rs601338) and *FUT3* genes (rs28362459, rs3894326, rs812936 and rs778986) on rotavirus-specific serum IgG antibody responses and neutralizing antibody responses to rotavirus Wa (P[8]) and ST3 (P[6]) strains in Swedish individuals (IgA competent or deficient). The rotavirus specific serum IgG levels and neutralizing antibody titers to the Wa strain (P[8]) of rotavirus were significantly higher in secretors than non-secretors ( $P < 0.001$ ), suggesting that secretors are more prone to P[8] rotavirus infections as compared to non-secretor individuals, independent of their IgA status. We thus suggest that distinct protection mechanisms against rotavirus might be engaged in IgA competent healthy controls, *FUT2* mediated, and in IgA deficient individuals, both *TLR3* and *FUT2* mediated.

### 4.3. PAPER III

In this study, *L. rhamnosus* GG, a well-known probiotic strain of *Lactobacillus*, was engineered to produce surface-anchored IgG-binding domains of protein G

which capture rotavirus-specific hyperimmune bovine colostrum (HBC) antibodies for protection against rotavirus. The combination therapy with engineered *L. rhamnosus* GG (PG3) and HBC was significantly more effective in reducing the prevalence, severity, and duration of diarrhea in a mouse pup model of RRV infection, in comparison to HBC alone or a combination of wild-type *L. rhamnosus* GG and HBC. This combination therapy can reduce the treatment costs, and the antibody capturing platform can further be applied to treat other gastrointestinal pathogens.

#### **4.4. PAPER IV**

In *Paper IV*, we have developed vectors including two expression cassettes in tandem for co-expression of two rotavirus- specific llama heavy chain variable fragment (VHH): ARP1 and ARP3. Engineered *L. paracasei* BL23 was shown to produce two antibody fragments (secreted or anchored), and bind to a broad range of rotavirus serotypes (including the human rotavirus strains 69M, Va70, F45, DS1, Wa and ST3 and simian rotavirus strains RRV and SA11) *in vitro*. This *Lactobacillus*-based co-expression platform might help reducing appearance of escape mutants, as the two VHH fragments are targeting distinct epitopes of rotavirus, and be used to produce other VHHs or proteins to target other gastrointestinal pathogens in future.

#### **4.5. PAPER V**

In this study, we have fused the mouse IgG1 Fc to ARP1 to improve the protective capacity of ARP1 *in vivo*. We have shown that the Fc-ARP1 fusion protein confers a significantly increased protection against rotavirus in a neonatal mouse model of rotavirus-induced diarrhea by reducing the prevalence, duration and severity of diarrhea and the viral load in small intestines of mouse pups. We suggest that the Fc part of immunoglobulins might be engaged in intracellular neutralization by involving Fc receptor activation.

## 5. DISCUSSION

Rotavirus infections affect young children all around the globe. The increase in severity of rotavirus induced diarrhea and susceptibility to rotavirus infections might be attributed to several factors (117) including malnutrition, low birth weight, cool season, dry climate, poor hygiene conditions, healthcare associated infections (outbreaks in neonatal intensive care units or hospital visits), infections with different co-circulating rotavirus genotypes, co-infections with other pathogens (HIV, *E. coli*, and *Salmonella*, etc.), immunodeficiency (X-linked agammaglobulinemia (XLA), common variable immunodeficiency (CVID), severe combined immunodeficiency (SCID), and IgA deficiency), and genetic factors (mutations in genes of TLR signaling pathway, and in *FUT2* and *FUT3* genes involved in the synthesis of HBGAs).

The two rotavirus vaccines, Rotarix<sup>TM</sup> and Rotateq<sup>TM</sup> are currently licensed for protection against rotavirus in infants. While rotavirus induced diarrhea might be referred to as a vaccine preventable disease in certain parts of the world (more than 85 % efficacy in countries in Europe, South and North America, and Australia), the vaccine efficacy is relatively low (40-60 %) in several other countries in Asia and Africa, where rotavirus is the major pediatric pathogen causing high mortality (accounts for a cumulative mortality of 85 %) (60, 61, 118). In addition to low vaccine efficacy issues in the most affected populations, the vaccine companies have notified plausible vaccine-associated contraindications for use in infants with XLA, CVID and SCID. Interestingly, immunization protocols in some countries suggest consultation with an immunologist for individuals with IgAD before vaccination. However, the fact that vaccination starts at the age of 6-12 (Rotateq<sup>TM</sup>), or 6-24 weeks (Rotarix<sup>TM</sup>) means that infants with immunodeficiency are not diagnosed prior to vaccination (median age for diagnosis of SCID: 4-7 months), putting the immunosuppressed infants with no family history of immunodeficiency into great danger for severe post-vaccination outcomes including acute diarrhea, shedding vaccine-acquired rotavirus strains, co-infection with pathogens including *E. coli* and *Salmonella*, and extended rotavirus shedding (up to 11 months) (67). Furthermore, there is



inadequate information on vaccine safety and efficacy in undiagnosed HIV infected children in developing countries, which also raises concerns for risk of developing post-vaccine symptoms.

The chronic wild-type and vaccine-acquired rotavirus infections in infants with SCID might be detrimental, causing chronic diarrhea, failure to thrive and early onset of infections. Early diagnosis of the disease and hematopoietic stem cell transplantation before onset of severe infections are therefore required (64, 119). The affected immunocompromised infants might also be the ones suffering from the lack of specific maternally derived secretory IgA antibodies in breast milk directed against the emerging serotypes of rotavirus (120). In addition, infants with primary immunodeficiency in the TLR signaling pathways have also been reported to suffer from chronic infections (121). Deficiencies in certain TLR-pathways, such as MyD88-IRAK4 and TLR3-Unc93b-TRAF3, confer predisposition to pyogenic bacteria and HSV encephalitis, respectively (122, 123), which might be due to reduced or impaired IFN production (124). Even though adults with compromised TLR signaling show no severe clinical outcomes, it might be different for children. More than 100 polymorphisms have been identified in the *TLR3* gene so far, some of which have functional implications due to the lack of TLR3 expression and reduced functionality (rs5743305 and rs3775291), such as impaired IFN- $\alpha$ / $\beta$  and IFN- $\lambda$  production. Patients with TLR3 deficiency have been shown to be susceptible to HSV. In *Paper I*, it has been shown that two polymorphisms (rs5743305 and rs3775291), causing impaired or reduced TLR3 expression or function, might also be a factor for susceptibility to rotavirus infection in immunodeficient, but not in immunocompetent individuals. As previously shown (44), elevated anti-rotavirus IgG antibody titers was shown in the IgA deficient as compared to the IgA-sufficient group. To determine the immunological basis for the elevated rotavirus-specific titers in individuals with IgAD, intragroup differences in IgAD individuals were analyzed. Interestingly, no change in anti-rotavirus IgG response was detected in IgA competent individuals with impaired *TLR3* gene.

However, individuals with combined IgA and TLR3 deficiency were presented with increased specific IgG titers as compared to individuals with impaired TLR3 only, suggesting an increased susceptibility to severe, chronic and/or recurrent rotavirus infection in individuals with both of these defects (44). Reduced TLR3 expression in neonatal mice has previously been shown to be associated with rotavirus susceptibility by Pott *et al.*, suggesting that a relatively under-developed immune system, due to young age or mutations, make individuals vulnerable to infections. Although, complete lack of TLR3 signaling can cause severe clinical outcomes in some individuals, there is no information about the impact of these polymorphisms at the population level.

The genetic variations at the population level might also influence susceptibility to rotavirus infections. Until recently, low vaccine responses in the developing world has been associated with a higher incidence of concomitant infections, larger rotavirus strain diversity, and poor health status. Recent *in vitro* studies have suggested that HBGAs (H type 1 and Lewis antigens) are putative receptors for rotavirus VP8\* (10, 16, 17). Therefore, we have studied the effect of inactivating polymorphisms in the *FUT2* (rs601338) and *FUT3* genes (rs28362459, rs3894326, rs812936 and rs778986), contributing to secretor and Lewis phenotypes, respectively, in the antibody response to rotavirus in Swedish individuals (*Paper II*). The rotavirus specific serum IgG titers and neutralizing antibody levels to the Wa strain (P[8]) of rotavirus were significantly higher in secretors (individuals with at least one functional *FUT2* gene) than in non-secretors (individuals with homozygous nonsense mutation in the *FUT2* gene), suggesting that secretors are more prone to rotavirus (P[8]) infections as compared to non-secretors (*Paper II*). This finding implies that the Lewis negative individuals, being approximately 22-32 % in Africa (21, 23), are most likely resistant to P[8] virus infection. However, both vaccine formulations are based on rotavirus with P[8] genotype, and meant to resolve rotavirus infections especially in the developing world including Africa. The resistance to P[8] rotavirus infections through non-secretor phenotype might clarify the reduction in efficacy of current P[8]-based vaccines used in the developing world, more particularly in sub-Saharan Africa. The reason of high efficacy of vaccines used

in developed part of the world could be because both the vaccine strain and circulating rotavirus strain match. Furthermore, the majority of the population is composed of secretors, whom can be infected with either of the wild-type and vaccine P[8] virus strains. Interestingly, rotavirus P[6] infections comprise approximately one third of all the characterized P types in Africa (7), suggesting that vaccines should be tailored according to genetic needs of each population: a P[6]-based vaccine might serve as an efficient vaccine therapy in Africa. Furthermore, alternative or complementary effective prophylactic and therapeutic passive immunotherapy regimens, providing a more rapid response against rotavirus infections are warranted.

Passive immunotherapy has been used in prevention and treatment of many diseases, especially in fields where antimicrobial treatments are inadequate (125). The importance of passive immunotherapy was recognized in the early 1890s in the area of infectious diseases, by showing that antibodies conferred protection against bacteria and toxins, which allowed use of serum derived from immunized animals (sheep or horse) for treatment of infectious diseases (126). Later on, serum therapy was abandoned due to hypersensitivity reactions against animal proteins, and replaced by antimicrobials and antibiotics. However, raised awareness in the generation and spread of drug-resistant microorganisms in the environment, formation of newly emerging microorganisms and impractical use of antimicrobial treatments in immunosuppressed individuals focused attention back to antibody-based immunotherapy, a safe and immediate course of action against infectious diseases.

The traditional method of generating polyclonal antibodies in a host is by injection of the antigen of interest and subsequent collection of serum, containing specific antibodies. Polyclonal antibodies, mainly IgG, recognize multiple epitopes on an antigen, which might enhance detection and broaden the applicability of a given therapy. Thus, the fact that colostrum from selected species contains a high concentration of antibodies for protection of newborns from diseases, another polyclonal antibody production method was introduced. Pregnant cows challenged with an antigen of interest can produce large amounts

of specific antibodies, comprised mostly of IgG. The bovine colostrum IgG antibodies, derived from hyperimmunized cows, have previously been successfully used for protection in rotavirus-infected children in large field studies (127).

Probiotic bacteria represent an attractive delivery system of proteins and antibody fragments in the gastrointestinal tract. They colonize the gastrointestinal tract, ensuring the stability and stable expression or display of specific proteins at the site of gastrointestinal infections. Use of probiotic bacteria contained in a commercial product, BioGaia® Probiotic drops (Ewopharma AG, Bulgaria), is free from side effects if administered to healthy infants, children and adults, as well as immunodeficient adults, thus lactobacilli-mediated therapy might be applied to individuals at any age and with almost any immunologic background, including individuals with immunodeficiency. *L. rhamnosus* GG is a well-studied probiotic strain of lactic acid bacteria in gastrointestinal tract infections. When administered to hospitalized children with diarrhea, the prevalence of diarrhea and symptomatic rotavirus infection were markedly reduced (128, 129). Oral *L. rhamnosus* GG therapy also reduced the severity, duration and prevalence of infantile diarrhea caused by rotavirus RRV infections in a neonatal mouse model (79). *L. rhamnosus* GG was previously combined with exogenously delivered soluble HBC antibodies in a neonatal mouse pup model. When combined with *L. rhamnosus* GG, a 10-fold lower dose of HBC antibodies (10 µg) significantly reduced all measures of diarrhea similar to soluble HBC alone (100 µg) (79). The probiotic bacteria could potentially be involved in induction of innate immunity (secretion of cytokines) and production of mucosal IgA antibodies, which might inhibit the viral replication. In *Paper III*, *L. rhamnosus* GG was engineered to produce surface-anchored IgG-binding domains of protein G (PG3), which capture exogenous rotavirus-specific hyperimmune bovine colostrum (HBC) antibodies, consisting mostly of IgG antibodies, for protection against rotavirus. The new combination therapy reduces the effective dose of HBC by 10 to 100 fold, and the effective *L. rhamnosus* GG dose by 10 fold, and may thus decrease treatment costs. The rotavirus specific HBC antibodies might neutralize virus in bacteria-displayed or

soluble form through distinct mechanisms. The dense multimeric display of IgG by PG3 might promote a higher avidity in binding and stronger agglutination compared to the same amount of soluble antibody. This might result in enhanced blocking and reduce infection of mucosal cells. The soluble antibodies might also contribute to virus neutralization by binding to free rotaviral particles or to available epitopes of rotavirus, which are stabilized by PG3. Free HBC-IgG might also bind to the mouse neonatal Fc receptors (FcRn) (130) and neutralize virus intracellularly. PG3 would also be expected to extend the half-life of the HBC antibodies in the gastrointestinal tract.

Antibody-based therapy has a long history of reliable use, however high cost incurred by production of polyclonal antibodies have re-directed the research to focus on exploration of new highly efficient antibodies and antibody fragments derived from different sources (95, 96, 131, 132). The discovery of VHH antibody fragments is one of the novel advances in antibody therapy. VHHs are naturally available, thermostable, safe and small antibody fragments derived from *Camelidae* heavy chain antibodies. VHH antibody libraries have been generated from llamas immunized with rotavirus and shown to be cross-reactive against various G and P types. Rotavirus specific VHHs, including ARP1 and ARP3, were selected in low pH and in the presence of pepsin, providing stability during passage from the stomach to the gastrointestinal tract. Both ARP1 and ARP3 recognize dimeric VP6 protein of rotavirus (49), suggesting that they are most likely detecting structurally available, cryptic epitopes of virus, which might not be recognizable to conventional antibodies. Due to the small size, the rotavirus specific VHH antibody fragments can be produced by several organisms including yeast, lactic acid bacteria and mammalian cells, and be delivered as a food-grade product (expression in rice). Yeast produced VHH fragments (ARP1) were found to be safe in a Phase III clinical trial in children (98), and the rotavirus specific activity of rice-ARP1 did not change when boiled for 5 minutes (133), suggesting a high stability when passing through the harsh conditions of the stomach *in vivo*. The reasons for expressing and delivering antibodies or antibody fragments in organisms, involving probiotic lactic acid

bacteria, are to provide stability and improved activity, and reduce the production and storage expenses of the antibody therapy.

Another strain of *Lactobacillus*, *L. paracasei* BL23, was used to express VHH antibody fragments (*Paper IV*). Engineered lactobacilli producing surface-anchored ARP1 and ARP3 were previously generated and shown to be effective in a mouse model of rotavirus infection. The production of one VHH fragment targeting a single epitope has limitations due to reduced cross-reactivity to circulating viral serotypes and potentially emerging viral escape mutants. In contrast, targeting multiple epitopes might improve the efficacy due to an increased activity towards a broad range of viral serotypes and a less likelihood of accumulating viral escape mutants. The aim of this study was to generate co-expression vectors for the production of two rotavirus-specific ARP1 and ARP3, which might increase the avidity for rotavirus binding and aggregation due to binding to distinct epitopes. Additionally, if the virus acquires mutation on the binding site of an antibody fragment, the other antibody fragment will retain its activity against the virus. ARP1 and ARP3 were produced in vectors by two different expression cassettes: one allowing secretion, the other surface-anchoring of antibody fragments. Secreted VHH(s) might neutralize the virus outside the cell by preventing cell attachment, as well as inside the cell if it enters bound to rotavirus (due to its small size). However, anchored VHHs can saturate the surface of bacteria (6000 molecules/bacterium) (134), and might induce a stronger aggregation and neutralization response. It was previously shown in mice that *L. paracasei* BL23 producing anchored ARP1 confer stronger reduction in rotavirus diarrhea than the secreted version (108). Expression of distinct rotavirus specific fragments in *Lactobacillus* could increase the chance of neutralization through binding to different epitopes of the virus. The *Paper IV* has shown for the first time that ARP1 and ARP3, produced by engineered lactobacilli and directed against different epitope specificity, can bind strongly to several human and simian rotavirus strains from distinct genotypes, suggesting a broader cross-reactivity to many G/P combinations, thus representing an alternative therapy for individuals in the developing world who are infected mostly with rare rotavirus genotypes.

In addition to neutralizing activity outside the cells, Fc-mediated responses can be induced for an improved intracellular neutralization, as suggested by *Paper V*. The ARP1 fragment, which was extensively studied in our previous studies, was fused to a mouse IgG1 Fc to evaluate the *in vivo* effect of Fc and potential involvement of Fc effector functions. When fused to Fc, the anti-rotavirus effect of ARP1 was dramatically increased, not related to bivalency of ARP1, as bivalent ARP1 did not show any sign of protection against rotavirus diarrhea in mouse pups at the tested concentrations (provided that equal ARP1 molecules are given in each treatment). The N434F mutation was recently described to dramatically reduce the *in vitro* binding of IgG Fc to TRIM21, a cytosolic Fc receptor (135). This mutation was introduced into the Fc of the Fc-ARP1 fusion protein in *Paper V*. The infant mice treated with mutated Fc<sub>N434F</sub>-ARP1 showed a slightly lower protection against rotavirus than the mice treated with Fc-ARP1, suggesting either TRIM21 might not be involved in protection against rotavirus to a greater extent or the mutation did not function as expected based on *in vitro* studies. A pull-down assay will be performed in the future to evaluate the binding of Fc-ARP1 and mutated Fc<sub>N434F</sub>-ARP1 to TRIM21.

In addition to TRIM21, other Fc receptors (FcRn and FcγR) might also play role in intracellular rotavirus neutralization. Even though they also detect antibody-virus complex through the Fc part of the antibody, their binding site is not exactly the same as the TRIM21 receptor binding site, which might explain why the mutation did not greatly affect the *in vivo* response. Although the mechanism by which the Fc receptor can greatly improve protection is not known, a clear Fc-mediated response initiated with the Fc part of llama-like antibody (Fc-ARP1) was shown in a neonatal mouse model of rotavirus diarrhea. In order to activate different cellular responses for an enhanced anti-rotavirus response, more than one therapy approach might be considered for use in future.

## 6. FUTURE PROSPECTIVES

The development of *Lactobacillus* based delivery of antibody and antibody fragments can be useful during an epidemic of rotavirus infections in developing countries or in hospital acquired infections. This treatment would particularly be useful for individuals who do not fit in the recruitment criteria for vaccine therapy, or simply are not responding to vaccines well.

For large-scale production of engineered *Lactobacillus*, the bacteria can be grown in fermentors, and stored and distributed in several forms of food: yoghurt, milk powder, etc. To avoid using antibiotic resistance markers and dissemination of genes, the gene encoding the specific antibody fragment can be inserted in the genome of *Lactobacillus* as previously described (134). In preparation for a safety clinical trial, the ARP1 encoding gene was recently inserted into the genome of *L. rhamnosus* GG by site specific recombination, and the thymidine gene was deleted, allowing conditional growth of bacteria. This *Lactobacillus* delivery system, if safe in Phase I, and safe and efficient in Phase II-III clinical trials, can be used to produce multivalent ARPs for an improved protection against rotavirus. The advantages of using genetically modified lactobacilli are to provide a treatment combining safety, low cost of production, long term stable conservation in non-refrigerated conditions, rapid distribution, easy administration and adequate long term protection.

Future therapeutic strategies should probably trigger different modes of immune action in. The gene encoding the sequence of protein G could be integrated into the genome of *L. rhamnosus* GG. This modified *Lactobacillus* can be further combined with HBC-IgG or a mixture of Fc-ARP1 and Fc-ARP3. In addition, even though the full size Fc-ARP1 antibody cannot be expressed by lactobacilli, ARP1 could instead be fused to a VHH against the FcRn or FcγR in order to improve intracellular neutralization. This dimeric VHH could subsequently be expressed as a secreted protein in lactobacilli. The efficiency in transcytosis might be further improved through receptor clustering by using multivalent VHHs against the receptor. VHHs targeting the polymeric immunoglobulin receptor were recently used to deliver therapeutic molecules from basolateral to



apical side of mammalian cells *in vitro* (136), showing the potential of VHHs as carriers across epithelia.

Alternatively, engineered lactobacilli delivering multimeric VHHs against a panel of enteric pathogens can be used for simultaneous treatment against several gastrointestinal microbes threatening the lives of children in the developing world. The children with a family history of immunodeficiency, at risk to develop chronic rotavirus infections, can be provided with engineered lactobacilli producing cytokines, involved in clearance of rotavirus (137).

In the future, personalized therapy will be provided for each individual. Neonatal screening for severe forms of immunodeficiency, introduced in selected states in the USA (138), may provide early diagnosis and allow individualized therapy prior to rotavirus vaccination.

## 7. CONCLUSIONS

**Paper I.** Polymorphisms in *TLR3* gene are associated with increased levels of rotavirus-specific IgG antibody responses in individuals with IgA deficiency, suggesting an increased susceptibility to rotavirus infection in individuals with both *TLR3* and IgA deficiency.

**Paper II.** Individuals with intact secretor (*FUT2*) gene have elevated rotavirus-specific IgG and neutralizing antibody responses, independent of their IgA status, suggesting that human blood group antigens serve as susceptibility factors to rotavirus infections.

**Paper III.** A combination therapy, consisting of engineered *L. rhamnosus* GG expressing surface anchored IgG binding domains of protein G, and soluble anti-rotavirus HBC antibodies markedly reduced the prevalence, severity and duration of diarrhea in a mouse pup model of RRV infection.

**Paper IV.** *L. paracasei* BL23 co-expressing rotavirus-specific llama VHH fragments (ARP1 and ARP3) in secreted and cell wall-anchored forms, bind to variable human and simian rotavirus strains with a broad range of genotypes.

**Paper V.** Fusing ARP1 to the Fc fragment of mouse IgG1 (Fc-ARP1) increases Fc-mediated neutralization of rotavirus in a neonatal mouse model.

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